# This Page Is Inserted by IFW Operations and is not a part of the Official Record

### BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

## IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents will not correct images, please do not report the images to the Image Problem Mailbox.

Axit p sequence tollowing Ser<sup>208</sup> and occurs within the domain of Aut p that shows incrmology with hIDE (14). To delete the complete STE23 sequence and create the ste23A::URA3 mutation, polymerase chain reaction (PCR) primers (5'-TCGGAAGACCTCAT-TCTTGCTCATTTTGATATTGCTC-TGTAGATTG-TACTGAGAGTGCAC-3': 8nd 5'-GCTACAAACAGC-GTCGACTTGAATGCCCCGACATCTTCGACTGT-GOGGTATTTCACACCG-3") were used to amplify the URA3 sequence of pRS316, and the reaction product was transformed into yeast for one-step gene replacement (R. Rotinstein, Methods Enzymol. 194, 281 (1991)]. To create the aut 1 A: LEU2 mutation contained on p114, a 5.0-kb Sal I fragment from pAXL1 was cloned into pUC19, and an internal 4.0-kb Hpa I–Xho I tragment was replaced with a LEU2 tragment. To construct the ste23A-t FLI2 allele (a deletion corresponding to 931 amino acids) carried on p153, a LEUZ tragment was used to reptace the 2.8-kb Pml I-Ect136 II tragment of STE23, which occurs within a 6.2-kb Hind II-Bgl II genomic tragment carried on pSP72 (Promega). To create YEpMFA1, a 1.6-kb Barn HI tragment containing MFA1, trom pKK16 (K. Kuchler, R. E. Sterne, J. Thorner, EMBO J. 8, 3973 (1989)], was tigated into the Barn HI site of YEp351 [J. E. Hill, A. M. Myers, T. J. Koerner, A. Tzagoloff, Yeast 2, 163 (1986)].

- 24. J. Chant and I. Herskowitz, Cell 65, 1203 (1991).
- 25. B. W. Matthews, Acc. Chem. Res. 21, 333 (1988).
- K. Kuchler, H. G. Dohlman, J. Thorner; J. Cell Biol. 120, 1203 (1993); R. Kolling and C. P. Hollenberg, EMBO J. 13, 3261 (1994); C. Berkower, D. Loayza, S. Michaelis, Mol. Biol. Cell 5, 1185 (1994).
- Michaels, Mil. Biol. Cell 5, 1130 (1994).
   A. Bender and J. R. Pringle, Proc. Natl. Acad. Sci. U.S.A. 86, 9976 (1989); J. Chant, K. Corrado, J. R. Pringle, I. Herskowitz, Cell 65, 1213 (1991); S. Powers, E. Gonzales, T. Christensen, J. Cubert, D. Broek, Ibid., p. 1225; H. O. Park, J. Chant, I. Herskowitz, Nature 365, 269 (1993); J. Chant, Trends Genel. 10, 328 (1994); \_\_\_\_\_\_ and J. R. Pringle, J. Cell Biol. 129, 751 (1995); J. Chant, M. Mischke, E. Mitchell, I. Herskowitz, J. R. Pringle, biol., p. 767.
- 28. G. F. Sprague Jr., Methods. Enzymol. 194, 77
- Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Aso; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; O, Gin; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
- 30. A W303 1A derivative, SY2625 (MATe ura3-1 leu2-3, 112 trp1-1 ade2-1 can1-100 sst1Δ mfa2Δ::FUS1-lacZ his34::FUS1-HIS3), was the parent strain for the mutant search. SY2625 derivatives for the mating assays, secreted pheromone assays, and the putse-chase experiments included the following strains: Y49 (ste22-1), Y115 (mfa1&:1EUZ), Y142 (axf1::URA3), Y173 (ad1 A::LBU2), Y220 (ad1::URA3 ste23 A::URA3), Y221 (ste23A::URA3), Y231 (ax1A::LEU2 ste23A::LEU2), and Y233 (ste23A::LEU2), MATa derivatives of SY2625 included the following strains: Y199 (SY2625 made MATa), Y278 (ste22-1), Y195 (mfa1a::LEUZ), Y196 (exi1a::LEUZ), and Y197 (axt1::URA3). The EG123 (MATa leu2 ura3 trp1 can1 his4) genetic background was used to create a set of strains for analysis of bud site selection. EG123 defivatives included the following strains: Y175 (axi1\(\text{\alpha}\):\LEU2), Y223 (axi1\(\text{\alpha}\):\LEU2), Y234 (sta23\(\text{\alpha}\):\LEU2), and Y272 (axi1\(\text{\alpha}\):\LEU2 ste23\(\text{\alpha}\):\LEU2). MATa derivatives of EG123 included the following strains: Y214 (EG123 made MATa) and Y293 (aud) A::LEU2). All strains were generated by means of standard genetic or molecular methods involving the appropriate constructs (23). In particular, the aid 1 ste23 double mutant strains were created by crossing of the appropriate MATa sta23 and MATa aud1 mutants, followed by sponulation of the resultant dip-loid and isolation of the double mutant from nonparental di-type tetrads. Gene disruptions were confirmed with either PCR or Southern (DNA) analysis.
- p129 is a YEp352 [J. E. Hill, A. M. Myers, T. J. Koerner, A. Tzagotoff, Yesst 2, 163 (1986)] plasmid containing a 5.5-kb Sal I tragment of pAVL 1, p151 was derived from p129 by insention of a linker at the Bgl II site within AVL 1, which led to an in-terme insention of the hermagglutnin (HA) epitope (DCYPYDVPDYA) (29) between amino acids 854 and 855 of the AVL 1 prod-

uct. pC225 is a KS+ (Stratagene) prasmid containing a 0.5-kb Barn HI-Sst I tracment from pAXL1, Substitution mutations of the proposed active site of AxII p were created with the use of pC225 and site-specific mutagenesis involving appropriate synthetic oligonu-cleotoes (ax1-H68A, 5'-GTGCTCACAAAGCGCT-GOCAAACOGGC-3'; BX1-E71A, 5'-AAGAATCAT-GTGCGCACAAAGGTGCGC-3'; and auti-E71D, 5'-AAGAATCATGTGATCACAAAGGTGCGC-3"). mutations were confirmed by sequence analysis. Alter mutagenesis, the 0.4-kb Barn HI-Msc I tragment from the mutagenized pC225 plasmids was transterred into pAXL1 to create a set of pRS316 plasmids carrying different AXL1 alleles, p124 (ax1-H68A), p130 (ax11-E71A), and p132 (ax11-E71D). Similarly, 8 set of HA-tagged alleies carned on YEp352 were created after replacement of the p151 Barn HI-Msc I tragment, to generate p161 (ax11-E71A), p162 (ax11-

MSAN, and p163 (ax11-E710).

32. We thank J. Becker and S. Michaelis for providing elector entibodies; S. Michaelis for discussing unpublished results and helping with the pulse-chase experiments; J. Brown, J. Chart, and S. Sanders for their input concerning buo site selection experiments; M. Raymond, F. Taminol, and M. Whiteway for blasmics; M. Marra for providing the STE23 genomic tragment; and H. Bussey, J. Brown, N. Davis, T. Favero, C. de Hoog, and S. Kim for comments on the manuscript. Supported by a grant to C.B. from the Natural Scences and Engineering Research Council of Canada. Support for M.N.A. was from a California Tobacco-Retated Disease Research Program postocctoral tellowship (AFT-0083).

22 June 1995; accepted 21 August 1995

## Quantitative Monitoring of Gene Expression Patterns with a Complementary DNA Microarray

Mark Schena,\* Dari Shalon,\*† Ronald W. Davis, Patrick O. Brown‡

A high-capacity system was developed to monitor the expression of many genes in parallel. Microarrays prepared by high-speed robotic printing of complementary DNAs on glass were used for quantitative expression measurements of the corresponding genes. Because of the small format and high density of the arrays, hybridization volumes of 2 microliters could be used that enabled detection of rare transcripts in probe mixtures derived from 2 micrograms of total cellular messenger RNA. Differential expression measurements of 45 Arabidopsis genes were made by means of simultaneous, two-color fluorescence hybridization.

The temporal, developmental, topographical, histological, and physiological patterns in which a gene is expressed provide clues to its biological role. The large and expanding database of complementary DNA (cDNA) sequences from many organisms (1) presents the opportunity of defining these patterns at the level of the whole genome.

For these studies, we used the small flowering plant Arabidopsis thaliana as a model organism. Arabidopsis possesses many advantages for gene expression analysis, including the fact that it has the smallest genome of any higher eukaryote examined to date (2). Forty-five cloned Arabidopsis cDNAs (Table 1), including 14 complete sequences and 31 expressed sequence tags (ESTs), were used as gene-specific targets. We obtained the ESTs by selecting cDNA clones at random from an Arabidopsis cDNA library. Sequence analysis revealed that 28 of the 31 ESTs matched sequences in the database (Table 1). Three additional cDNAs from other organisms served as controls in the experiments.

The 48 cDNAs, averaging ~1.0 kb. were amplified with the polymerase chain reaction (PCR) and deposited into individual wells of a 96-well microtiter plate. Each sample was duplicated in two adjacent wells to allow the reproducibility of the arraying and hybridization process to be tested. Samples from the microtiter plate were printed onto glass microscope slides in an area measuring 3.5 mm by 5.5 mm with the use of a high-speed arraying machine (3). The arrays were processed by chemical and heat treatment to attach the DNA sequences to the glass surface and denature them (3). Three arrays, printed in a single lot, were used for the experiments here. A single microtiter plate of PCR products provides sufficient material to print at least 500 arrays.

Fluorescent probes were prepared from total Arabidopsis mRNA (4) by a single round of reverse transcription (5). The Arabidopsis mRNA was supplemented with human acetylcholine receptor (AChR) mRNA at a dilution of 1:10,000 (w/w) before cDNA synthesis, to provide an internal standard for calibration (5). The resulting fluorescently labeled cDNA mixture was hybridized to an array at high stringency (6) and scanned

M. Schena and R. W. Davis, Department of Biochemistry, Beckman Center, Stanford University Medical Center, Stanford, CA 94305, USA.

D. Shalon and P. O. Brown, Department of Biochemistry and Howard Hughes Medical Institute, Beckman Center, Stanford University Medical Center, Stanford, CA 94305, USA.

<sup>\*</sup>These authors contributed equally to this work. Present address: Synteni, Palo Alto, CA 94303, USA. \*To whom correspondence should be addressed. Email: pbrown@cmgm.stanford.edu

with a laser (3). A high-sensitivity scan gave signals that saturated the detector at nearly all of the Arabidopsis target sites (Fig. 1A). Calibration relative to the AChR mRNA standard (Fig. 1A) established a sensitivity limit of ~1:50,000. No detectable hybridization was observed to either the rat glucocorticoid receptor (Fig. 1A) or the yeast TRP4 (Fig. 1A) targets even at the highest scanning sensitivity. A moderate-sensitivity scanning sensitivity.

of the same array allowed linear detection of the more abundant transcripts (Fig. 1B). Quantitation of both scans revealed a range of expression levels spanning three orders of magnitude for the 45 genes tested (Table 2). RNA blots (7) for several genes (Fig. 2) comborated the expression levels measured with the microarray to within a factor of 5 (Table 2).

Differential gene expression was investi-

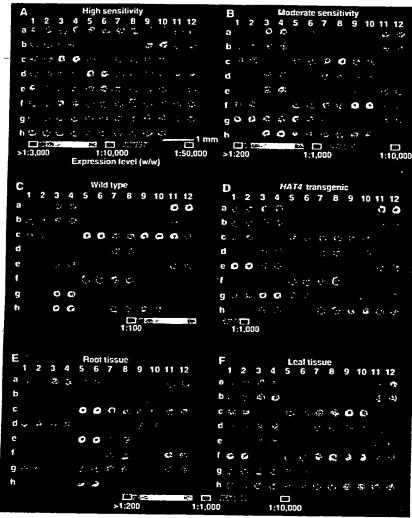


Fig. 1. Gene expression monitored with the use of cDNA microarrays. Fluorescent scans represented in pseudocolor correspond to hybridization intensities. Color bars were calibrated from the signal obtained with the use of known concentrations of human AChR mRNA in independent experiments. Numbers and letters on the axes mark the position of each cDNA. (A) High-sensitivity fluorescein scan after hybridization with fluorescein-labeled cDNA derived from wild-type plants. (B) Same array as in (A) but scanned at moderate sensitivity. (C and D) A single array was probed with a 1:1 mixture of fluorescein-labeled cDNA from wild-type plants and lissamine-labeled cDNA from HAT4-transgenic plants. The single array was then scanned successively to detect the fluorescein fluorescence corresponding to mRNA from wild-type plants (C) and the lissamine fluorescence corresponding to mRNA from HAT4-transgenic plants (D). (E and F) A single array was probed with a 1:1 mixture of fluorescein-labeled cDNA from root tissue and lissamine-labeled cDNA from leaf tissue. The single array was then scanned successively to detect the fluorescein fluorescence corresponding to mRNAs expressed in leaves (F).

gated with a simultaneous, two-color hybridization scheme, which served to minimize experimental variation inherent in the comparison of independent hybridizations. Fluorescent probes were prepared from two mRNA sources with the use of reverse transcriptase in the presence of fluorescein- and lissamine-labeled nucleotide analogs, respectively (5). The two probes were then mixed together in equal proportions, hybridized to a single array, and scanned separately for fluorescein and lissamine emission after independent excitation of the two fluorophores (3).

To test whether overexpression of a single gene could be detected in a pool of total Arabidopsis mRNA, we used a microarray to analyze a transgenic line overexpressing the single transcription factor HAT4 (8). Fluorescent probes representing mRNA from wild-type and HAT4-transgenic plants were labeled with fluorescein and lissamine, respectively; the two probes were then mixed and hybridized to a single array. An intense hybridization signal was observed at the position of the HAT4 cDNA in the lissamine-specific scan (Fig. 1D), but not in the fluorescein-specific scan of the same array (Fig. 1C). Calibration with AChR mRNA added to the fluorescein and lissamine cDNA synthesis reactions at dilutions of 1:10,000 (Fig. 1C) and 1:100 (Fig. 1D), respectively, revealed a 50-fold elevation of HAT4 mRNA in the transgenic line relative to its abundance in wild-type plants (Table 2). This magnitude of HAT4 overexpression matched that inferred from the Northern (RNA) analysis within a factor of 2 (Fig. 2 and Table 2). Expression of all the other genes monitored on the array differed by less than a factor of 5 between HAT4transgenic and wild-type plants (Fig 1, C

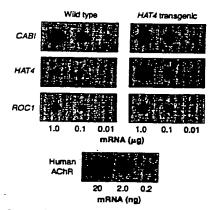


Fig. 2. Gene expression monitored with RNA (Northern) blot analysis. Designated amounts of mRNA from wild-type and HAT4-transgenic plants were spotted onto nylon membranes and probed with the cDNAs indicated. Purified human AChR mRNA was used for calibration.

and D, and Table 2). Hybridization of fluorescein-labeled glucocorticoid receptor cDNA (Fig. 1C) and lissamine-labeled TRP4 cDNA (Fig. 1D) verified the presence of the negative control targets and the lack of optical cross talk between the two fluorophores.

To explore a more complex alteration in expression patterns, we performed a second two-color hybridization experiment with fluorescein- and lissamine-labeled probes prepared from root and leaf mRNA, respectively. The scanning sensitivities for the two fluorophores were normalized by matching the signals resulting from AChR

mRNA, which was added to both cDNA synthesis reactions at a dilution of 1:1000 (Fig. 1, E and F). A comparison of the scans revealed widespread differences in gene expression between root and leaf tissue (Fig. 1, E and F). The mRNA from the light-regulated CABI gene was ~500-fold more abundant in leaf (Fig. 1F) than in root tissue (Fig. 1E). The expression of 26 other genes differed between root and leaf tissue by more than a factor of 5 (Fig. 1, E and F).

The HAT4-transgenic line we examined has elongated hypocotyls, early flowering, poor germination, and altered pigmentation (8). Although changes in expression were

Table 1. Sequences contained on the cDNA microarray. Shown is the position, the known or putative function, and the accession number of each cDNA in the microarray (Fig. 1). All but three of the ESTs used in this study matched a sequence in the database. NADH, reduced form of nicotinamide adenine dinucleotide; ATPase, adenosine triphosphatase; GTP, guanosine triphosphate.

Position	cDNA	Function	Accession number
a1, 2	AChR	Human AChR	•
a3, 4	EST3	Actin	H36236
a5, 6	EST6	NADH dehydrogenase	Z27010
a7, 8	AAC1	Actin 1	
a9, 10	EST12	Unknown	M20016
a11, 12	EST13	Actin	U36594†
b1, 2	CABI	Chlorophyll a/b binding	T45783
b3, 4	EST17	Phosphoglycerate kinase	M85150
b5, 6	GA4	Gibberellic acid biosynthesis	T44490
b7, 8	EST19	Unknown	L37126
b9, 10	GBF-1	G-box binding factor 1	U36595†
b11, 12	EST23	Elongation factor	X63894
c1, 2	EST29	Aldolase	X52256 T04477
c3, 4	GBF-2	G-box binding factor 2	
c <u>5,</u> 6	EST34	Chloroplast protease	X63895
c7, B	EST35	Unknown	R87034
c9, 10	EST41	Catalase	T14152
11, 12	rGR	Rat glucocorticoid receptor	T22720
11, 2	EST42	Unknown	M14053
13, 4	EST45	ATPase	U36596†
15, 6	HAT1	Homeobox-leucine zipper 1	J04185
17, 8	EST46	Light harvesting complex	U09332
9, 10	EST49	Unknown	T04063
11, 12	HAT2	Homeobox-leucine zipper 2	T76267
1, 2	HAT4	Homeobox-leucine zipper 4	U09335
3, 4	EST50	Phosphoribulokinase	M90394
5, 6	HAT5	Homeobox-leucine zipper 5	T04344
7, 8	EST51	Unknown	M90416
9, 10	HAT22	Homeobox-leucine zipper 22	<b>Z33675</b>
11, 12	EST52	Oxygen evolving	U09336
, 2	EST59	Unknown	T21749
, 4	KNAT1	Knotted-like harneobax 1	Z34607
, 6	EST60	RuBisCO small subunit	U14174
. 8	EST69	Tempolation of a service of	X14564
. 10	PPH1	Translation elongation factor	T42799
1, 12	EST70	Protein phosphatase 1	U34803
, 2	EST75	Unknown	T44621
. 4	EST78	Chloroplast protease	T43698
, 6	ROC1	Unknown	R65481
. 8	EST82	Cyclophilin	L14844
10	EST83	GTP binding	X59152
1, 12	EST84	Unknown	Z33795
2	EST91	Unknown	T45278
4	EST96	Unknown	T13832
6	SAR1	Unknown	R64816
8		Synaptobrevin	M90418
10	EST100	Light narvesting complex	Z18205
, 12	EST103	Light harvesting complex	X03909
	TRP4	Yeast tryptophan biosynthesis	X04273

\*Proprietary sequence of Stratagene (La Jolla, California).

tNo match in the database; novel EST.

observed for HAT4, large changes in expression were not observed for any of the other 44 genes we examined. This was somewhat surprising, particularly because comparative analysis of leaf and root tissue identified 27 differentially expressed genes. Analysis of an expanded set of genes may be required to identify genes whose expression changes upon HAT4 overexpression; alternatively, a comparison of mRNA populations from specific tissues of wild-type and HAT4-transgenic plants may allow identification of downstream genes.

At the current density of robotic printing, it is feasible to scale up the fabrication process to produce arrays containing 20,000 cDNA targets. At this density, a single array would be sufficient to provide gene-specific targets encompassing nearly the entire repertoire of expressed genes in the Arabidopsis genome (2). The availability of 20,274 ESTs from Arabidopsis (1, 9) would provide a rich source of templates for such studies.

The estimated 100,000 genes in the human genome (10) exceeds the number of Arabidopsis genes by a factor of 5 (2). This modest increase in complexity suggests that similar cDNA microarrays, prepared from the rapidly growing repertoire of human ESTs (1), could be used to determine the expression patterns of tens of thousands of human genes in diverse cell types. Coupling an amplification strategy to the reverse transcription reaction (11) could make it feasible to monitor expression even in minute tissue samples. A wide variety of acute and chronic physiological and pathological conditions might lead to characteristic changes in the patterns of gene expression in peripheral blood cells or other easily sampled tissues. In concert with cDNA microarrays for monitoring complex expression patterns, these tissues might therefore serve as sensitive in vivo sensors for clinical diagnosis. Microarrays of cDNAs could thus provide a useful link between human gene sequences and clinical medicine.

Table 2. Gene expression monitoring by microarray and RNA blot analyses; tg, HAT4-transgenic. See Table 1 for additional gene information. Expression levels (w/w) were calibrated with the use of known amounts of human AChR mRNA. Values for the microarray were determined from microarray scans (Fig. 1); values for the RNA blot were determined from RNA blots (Fig. 2).

Gene	Expression level (w/w)	
	Microarray	RNA blot
CABI CABI (tg) HAT4 HAT4 (tg) ROC1 ROC1 (tg)	1:48 1:120 1:8300 1:150 1:1200 1:260	1:83 1:150 1:6300 1:210 1:1800 1:1300

#### REFERENCES AND NOTES

- The current EST database (dbEST release 091495) from the National Center for Biotechnology Information (Bethesda, MD) contains a total of 322,225 entries, including 255,645 from the human genome and 21,044 from Arabidopsis. Access is available via the World Wide Web (http://www.ncbi.ntm.nth.pov).
- E. M. Meyerowitz and R. E. Pruitt, Science 229, 1214 (1985); R. E. Pruitt and E. M. Meyerowitz, J. Mol. Biol. 187, 169 (1986); L. Hwang et al., Pant J. 1, 367 (1991); P. Javis et al., Pant Mol. Biol. 24, 685 (1994); L. Le Guen et al., Mol. Gen. Genet. 245, 390 (1994).
- 3. D. Shalon, thesis, Stanford University (1995); and P. O. Brown, in preparation. Microarrays were fabricated on poly-L-lysine-coated microscope slides (Sigma) with a custom-built arraying machin fitted with one printing tip. The tip loaded 1 µl of PCR product (0.5 mg/ml) from 96-well microtiter plates and deposited ~0.005 µl per slide on 40 slides at a spacing of 500 µm. The printed slides were rehydrated for 2 hours in a humid chamber, snap-dried at 100°C for 1 min, rinsed in 0.1% SDS, and treated with 0.05% succinic anhydride prepared in buffer consisting of 50% 1-methyl-2-pyrrolidinone and 50% boric acid. The cDNA on the slides was denatured in distilled water for 2 min at 90°C immediately before use. Microarrays were scanned with a laser fluorescent scanner that contained a computer-controlled XY stage and a microscope objective. A mixed gas, multiline laser allowed sequential excitation of the two fluorophores. Emitted light was split according to wavelength and detected with two photomustiplier tubes. Signals were read into a PC with the use of a 12-bit analog-to-digital board. Additional details of microarray fabrication and use may be obtained by means of e-mail (pbrown@cmgm, stanford.edu).
- F. M. Ausubel et al., Eds., Current Protocots in Molecular Biology (Greene & Wiley Interscience, New York, 1994), pp. 4.3.1–4.3.4.
- Polyadenylated (poly(A)\*) mRNA was prepared from total RNA with the use of Oilgotex-oT resin (Qiagen). Reverse transcription (RT) reactions were carried out with a StrataScript RT-PCR kit (Stratagene) modified as follows: 50-µl reactions contained 0.1 µg/µl of Arabidopsis mRNA, 0.1 ng/µl of human AChR mRNA, 0.05  $\mu g/\mu l$  of oligo(dT) (21-mer), 1 $\times$  first strand buffer, 0.03 U/ $\mu l$  of ribonuclease block, 500 µM deoxyadenosine triphosphate (dATP), 500 µM deoxyguanosine triphosphate, 500 µM dTTP, 40 µM deoxycytosine triphosphate (dCTP), 40 µM fluprescein-12-dCTP (or issamine-5-dCTP), and 0.03 U/µI of StrataScript reverse transcriptase. Reactions were incubated for 60 min at 37°C, precipitated with ethanol, and resuspended in 10 µ of TE (10 mM tris-HCl and 1 mM EDTA, pH 8.0). Samples were then heated for 3 min at 94°C and chilled on ice. The RNA was degraded by adding 0.25 µl of 10 N NaOH tollowed by a 10-min incubation at 37°C. The same ples were neutralized by addition of 2.5 µl of 1 M tris-CI (pH 8.0) and 0.25 µl of 10 N HO and precipitated with ethanol. Pellets were washed with 70% ethanol, dried to completion in a speedvac, resuspended in 10 µl of H<sub>2</sub>O, and reduced to 3.0 µl in a speedvac. Puorescent nucleotide analogs were obtained from New England Nuclear (DuPont).
- 6. Hybridization reactions contained 1.0 µl of fluorescent cDNA synthesis product (5) and 1.0 µl of hybridization buffer (10x saine sodium citrate (SSC) and 0.2% SDS). The 2.0 µl probe mixtures were afiquoted onto the microarray surface and covered with cover stips (12 mm round). Arrays were transferred to a hybridization chamber (3) and incubated for 18 hours at 65°C. Arrays were washed for 5 min at room temperature (25°C) in low-stringency wash buffer (1x SSC and 0.1% SDS), then for 10 min at room temperature in high-stringency wash buffer (0.1x SSC and 0.1% SDS). Arrays were scanned in 0.1x SSC with the use of a fluorescence laser-scanning device (3).
- 7. Samples of poly(A)\* mRNA (4, 5) were spotted onto nylon membranes (Nytran) and crosslinked with uttraviolet light with the use of a Stratalinker 1800 (Stratagene). Probes were prepared by random priming with the use of a Prime-It 8 kit (Stratagene) in the presence of (PP)(ATP. Hybridizations were carned out according to the instructions of the manu-

- tacturer. Quantitation was performed on a Phosphorimager (Molecular Dynamics).
- M. Schena and R. W. Davis, Proc. Natl. Acad. Sci. U.S.A. 89, 3894 (1992); M. Schena, A. M. Lloyd, R. W. Davis, Genes Dev. 7, 367 (1993); M. Schena and R. W. Davis, Proc. Natl. Acad. Sci. U.S.A. 91, 8393 (1994).
- H. Hofte et al., Plant J. 4, 1051 (1993); T. Newman et al., Plant Physiol. 106, 1241 (1994).
- N. E. Morton, Proc. Natl. Acad. Sci. U.S.A. 88, 7474 (1991); E. D. Green and R. H. Waterston, J. Am. Med. Assoc. 266, 1966 (1991); C. Bettanne-Chantelot, Cell 70, 1059 (1992); D. R. Cox et al., Science 265, 2031 (1994).
- E. S. Kawasaki et al., Proc. Natl. Acad. Sci. U.S.A. 85, 5698 (1988).
- 12. The laser fluorescent scanner was designed and tabricated in collaboration with S. Smith of Stanford University. Scanner and analysis software was developed by R. X. Xia. The succinic anhydride reaction was suggested by J. Multigan and J. Van Ness of Derwin Motecuter Corporation. Thanks to S. Theologis, C. Somervile, K. Yamamoto, and members of the laborationes of R.W.D. and P.O.B. for critical comments. Supported by the Howard Hughes Medical Institute and by grants from NH [P21HG00450] (P.O.B.) and P37AG00198 (R.W.D.)] and from NSF (MCB9106011) (R.W.D.) and by an NSF graduate tellowship (D.S.). P.O.B. is an assistant investigator of the Howard Hughes Medical Institute.
  - 11 August 1995; accepted 22 September 1995

### Gene Therapy in Peripheral Blood Lymphocytes and Bone Marrow for ADA Immunodeficient Patients

Claudio Bordignon,\* Luigi D. Notarangelo, Nadia Nobili, Giuliana Ferrari, Giulia Casorati, Paola Panina, Evelina Mazzolari, Daniela Maggioni, Claudia Rossi, Paolo Servida, Alberto G. Ugazio, Fulvio Mavilio

Adenosine deaminase (ADA) deficiency results in severe combined immunodeficiency, the first genetic disorder treated by gene therapy. Two different retroviral vectors were used to transfer ex vivo the human ADA minigene into bone marrow cells and peripheral blood lymphocytes from two patients undergoing exogenous enzyme replacement therapy. After 2 years of treatment, long-term survival of T and B lymphocytes, marrow cells, and granulocytes expressing the transferred ADA gene was demonstrated and resulted in normalization of the immune repertoire and restoration of cellular and humoral immunity. After discontinuation of treatment, T lymphocytes, derived from transduced peripheral blood lymphocytes, were progressively replaced by marrow-derived T cells in both patients. These results indicate successful gene transfer into long-lasting progenitor cells, producing a functional multilineage progeny.

Severe combined immunodeficiency associated with inherited deficiency of ADA (1) is usually fatal unless affected children are kept in protective isolation or the immune system is reconstituted by bone marrow transplantation from a human leukocyte antigen (HLA)—identical sibling donor (2). This is the therapy of choice, although it is available only for a minority of patients. In recent years, other forms of therapy have been developed, including transplants from haploidentical donors (3, 4), exogenous enzyme replacement (5), and somatic-cell gene therapy (6–9).

We previously reported a preclinical model in which ADA gene transfer and expression

successfully restored immune functions in human ADA-deficient (ADA+) peripheral blood lymphocytes (PBLs) in immunodeficient mice in vivo (10, 11). On the basis of these preclinical results, the clinical application of gene therapy for the treatment of ADA SCID (severe combined immunodeficiency disease) patients who previously failed exogenous enzyme replacement therapy was approved by our Institutional Ethical Committees and by the Italian National Committee for Bioethics (12). In addition to evaluating the safety and efficacy of the gene therapy procedure, the aim of the study was to define the relative role of PBLs and hematopoietic stem cells in the long-term reconstitution of immune functions after retroviral vector-mediated ADA gene transfer. For this purpose, two structurally identical vectors expressing the human ADA complementary DNA (cDNA), distinguishable by the presence of alternative restriction sites in a nonfunctional region of the viral long-terminal repeat

(LTR), were used to transduce PBLs and bone

marrow (BM) cells independently. This pro-

cedure allowed identification of the origin of

C. Bordignon, N. Nobill, G. Ferrari, D. Maggioni, C. Rossi, P. Servida, F. Mavilio, Telethon Gene Therapy Program tor Genetic Diseases, DIBIT, Istituto Scientifico H. S. Raftaele, Mlan, Italy.

L. D. Notarangelo, E. Mazzolari, A. G. Ugazio, Department of Pediatrics, University of Brescla Medical School, Brescla. Italy.

G. Casorati, Unità di Immunochimica, DIBIT, Istituto Scientifico H. S. Reffaele, Milan, Italy. P. Panina, Roche Milano Ricerche, Milan, Italy.

<sup>\*</sup>To whom correspondence should be addressed.